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Joint Lecture with ACCP

$G_S\alpha$ Knockouts in Mice and Man

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[Key Words] G protein, genomic imprinting, Albright hereditary osteodystrophy, pseudohypoparathyroidism, hormone resistance

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Heterotrimeric G proteins are molecular switches in signal transduction pathways which couple cell surface receptors for a multitude of extracellular hormonal, biochemical and sensory signals to intracellular effector enzymes and ion channels. Each G protein is composed of an α, β and γ subunit[1]. The α-subunits bind guanine nucleotides and interact with specific receptors and effectors. The α-subunit for G_S ($G_S\alpha$) is ubiquitously expressed and couples multiple receptors to the stimulation of adenylyl cyclase, resulting in increased production of intracellular cyclic adenosine monophosphate (cAMP), and the opening of specific ion channels. The human $G_S\alpha$ gene *GNAS1* is a single copy gene which is located on chromosome 20 at 20q13[2] and the mouse homolog *Gnas* is located within a region in the distal portion of chromosome 2 which is syntenic to 20q13 in humans[3)4)].

The first insights into the role of G_S-coupled pathways in a whole animal model was provided by patients with Albright hereditary osteodystrophy (AHO), an autosomal dominant disorder characterized by short stature, obesity, specific skeletal defects referred to as brachydactyly, subcutaneous ossifications and mental defects[5]. Even within the same kindred AHO patients present in one of two ways: 1. with the somatic features of AHO alone (termed pseudopseudohypoparathyroidism [PPHP]); 2. with AHO plus multihormone (termed pseudohypoparathyroidism type Ia [PHP Ia]). PHP Ia patients demonstrate resistance to multiple hormones which raise intracellular cAMP in their target tissues through G_S-coupled pathways, such as parathyroid hormone (PTH), thyrotropin and the gonadotropins. However they do not appear to be resistant to all hormones which work through G_S, since they respond normally to vasopressin and adrenocortocotropin.

In PHP Ia patients the urinary cAMP response to administered PTH is markedly reduced[6] which localizes the biochemical defect to a signaling component proximal to cAMP generation (receptor, G_S or adenylyl cyclase). It was subsequently shown that most AHO patients have a ～50% deficiency of G_S in membranes isolated from various easily accessible cell types (eg. erythrocytes, fibroblasts, platelets, and transformed lymphoblasts) based on a functional reconstitution assay[7)～12)], and this decreased G_S bioactivity is often associated with a ～50% decrease in $G_S\alpha$ mRNA and protein expression[13)～15)]. Decreased G_S bioactivity and $G_S\alpha$ expression has been documented in both PHP Ia and PPHP patients[16)] and heterozygous inactivating mutations of *GNAS1* have been identified in multiple AHO kindreds. As predicted, related PHP Ia and PPHP patients have identical mutations[5)17)18)]. The role of G_S in hormone action and bone development, as well as other biological processes, is further underscored by the fact that *GNAS1* mutations which lead to constitutive activation of $G_S\alpha$ results in the McCune-Albright syndrome, a disorder with pleiotropic manifestations, including endocrine hyperfunction and fibrous dysplasia of bone[19)20)].

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AHO appears to be an autosomal dominant disorder with variable expressivity (PHP Ia vs. PPHP). It has remained unclear why some patients with heterozygous *GNAS1* mutations develop multihormone resistance (PHP Ia) while others do not (PPHP). It has also remained unclear why PHP Ia patients demonstrate resistance to some hormones (eg. PTH) but not to others (eg. vasopressin), even though they all activate G_S-coupled pathways[21]. The first clue to this enigma was provided by the observation that the presence or absence of multihormone resistance in an affected patient is determined by which parent transmitted the AHO trait[22][23]. Maternal transmission of AHO or a *GNAS1* mutation leads to offspring with PHP Ia while paternal transmission leads to offspring with PPHP. It was therefore suggested that *GNAS1* expression might be controlled by genomic imprinting, an epigenetic phenomenon by which one allele (paternal or maternal, depending on the specific gene) is poorly expressed[24]. Imprinting is associated with allele-specific DNA methylation differences and this may be critical in the initiation and maintenance of imprinting. Genetic alterations of imprinted genes result in several human developmental disorders, including Angelman and Prader-Willi syndromes and Beckwith-Wiedeman syndrome, and has been implicated in tumorigenesis.

If the *GNAS1* paternal allele is imprinted (poorly expressed) in hormone target tissues (eg. the proximal tubule, the renal target for PTH), then the presence of a null mutation in the maternal allele would drastically decrease $G_S\alpha$ expression due to imprinting of the paternal allele and mutation of the maternal allele, and this would lead to hormone resistance (PHP Ia). In contrast, a null mutation in the paternal allele should have little effect on $G_S\alpha$ expression since this allele is already poorly expressed due to the imprint, and therefore would not lead to hormone resistance (PPHP). Consistent with this model, the urinary cAMP response to exogenous PTH is markedly reduced in PHP Ia patients but normal in PPHP patients[16]. If *GNAS1* is imprinted, it would have to be in a tissue-specific manner, since there is no evidence for imprinting in various accessible tissues (ie. $G_S\alpha$ expression is equally decreased by 50% in both PHP Ia and PPHP patients) and the transcript encoding $G_S\alpha$ has been shown to be biallelically expressed in several fetal tissues[25]~[27]. Therefore, one might need to examine specific hormone target tissues to obtain direct evidence that *GNAS1* is imprinted in humans.

The mouse homolog *Gnas* on distal chromosome 2 maps within a region which is presumed to have one or more imprinted genes since maternal and paternal uniparental disomies (UPDs) of this region result in distinct and opposite phenotypes[3]. To directly examine the imprinting status of *Gnas*, we generated mice with an insertion of a neomycin-resistance cassette into the coding sequence of exon 2, thereby creating a *Gnas* null allele (GsKO)[28]. GsKO homozygotes (−/−) are embryonically lethal. Female heterozygotes were mated to normal males and normal females were mated with male heterozygotes to generate m−/+ and +/p− offspring, respectively, and these two groups of mice have distinct and abnormal phenotypes. Newborn m−/+ mice have wide, square shaped bodies with transient subcutaneous edema and higher birth weights. At 6-21 days after birth, the majority of these mice develop neurological abnormalities and subsequently die. In contrast, newborn +/p− mice have narrow bodies, arched backs, and lower birth weights, and lack subcutaneous edema. Most +/p− newborns fail to suckle, become hypoglycemic and die within hours after birth. M−/+ and +/p− mice continue to show other phenotypic differences, particularly in terms of fat mass accumulation, with m−/+ mice developing obesity and +/p− mice becoming thinner than normal. Interestingly, many of the early phenotypes of m−/+ and +/p− mice are quite similar to those described for mice with paternal and maternal UPD of the imprinted region, respectively[3], suggesting that *Gnas* is imprinted and that this locus is important in the generation of both abnormal UPD phenotypes.

Similar to the genetic pattern in AHO patients, m−/+, but not +/p− mice show evidence for renal

resistance to PTH[28]. Northern blot, Western blot and *in situ* hybridization studies all show that $G_s\alpha$ expression in renal proximal tubules is markedly reduced in m−/+, but not in +/p− mice[28], demonstrating that the paternal allele is imprinted in this tissue. The *Gnas* paternal allele is also imprinted in brown and white adipose tissue, perhaps explaining the differences in lipid metabolism observed in these two groups of mice. As previously noted above, PHP Ia patients are resistant to some hormones (eg. PTH) but not to others (eg. vasopressin) which activate G_s. One possible explanation for this observation is that the $G_s\alpha$ gene is imprinted in a tissue-specific manner and is therefore not imprinted in the target tissues for some hormones, such as vasopressin. If this is the case, then all heterozygotes should generate a partial ~50% cAMP response to vasopressin due to haploinsufficiency of $G_s\alpha$. A partial cAMP response is often sufficient to produce a maximal physiological response, as has been shown for glucagon and isoproterenol in PHP Ia patients[29)~31)], and therefore hormone resistance may not be obvious. Maximal urinary concentrating ability to dehydration (a measure of maximal vasopressin response) is normal in m−/+ mice[28]. $G_s\alpha$ mRNA and protein expression in the renal inner medulla (the site of action for vasopressin) is equally reduced by ~50% in both m−/+ and +/p− mice, consistent with lack of imprinting in this tissue. Therefore tissue-specific imprinting of *Gnas* likely explains the variable and tissue-specific pattern of hormone resistance present in GsKO mice.

It seems likely that the variable presentation of AHO (PHP Ia vs. PPHP) and the tissue-specific nature of hormone resistance in PHPIa results from tissue-specific imprinting of *GNAS1*. This model could also explain the observation that PHP Ia patients have reduced PTH actions in the proximal tubule (leading to decreased 1,25 dihydroxyvitamin D synthesis and increased phosphate reabsorption) while its actions in the distal nephron (ie. the calcium reabsorption response) are maintained[32]. However, direct evidence for allelic differences in $G_s\alpha$ expression have been lacking[25)~27)], probably since this imprinting effect is limited to a small number of tissues in humans.

While *GNAS1* was originally thought to contain only 13 exons (numbered 1-13) encoding the $G_s\alpha$ protein[33], more recently it has been shown to be a much more complex gene with multiple alternative promoters and upstream exons splicing into exon 2, resulting in multiple alternative transcripts and gene products. One alternative transcript formed by the splicing of an alternative upstream exon (located 35 kB upstream of exon 1) into exon 2 encodes an alternative form of $G_s\alpha$, named XLαs, which has the conserved regions required for guanine nucleotide binding but also includes an extra-long amino-terminal extension[26)34)]. Another alternative transcript results from the splicing of a different alternative upstream exon (located 11 kB upstream to the XLαs exon) to exon 2[27]. This mRNA encodes NESP55, a member of the chromogranin family[35], which is structurally unrelated to the G protein α-subunit family. The entire NESP55 protein sequence is encoded in the alternative upstream exon and the properly spliced exons 2 through 13 are within the 3' untranslated region of the NESP55 transcript. The expression of both XLαs and NESP55 is limited primarily to neuroendocrine tissues and they probably play a role in the regulation of secretion. Another alternative promoter has been mapped ~2.5kB upstream of exon 1, but the resulting transcripts probably do not encode a functional protein[36)37)]. In summary, the $G_s\alpha$ genes have four or more promoters resulting in multiple alternative transcripts formed by splicing of alternative upstream exons into exon 2.

While there is as yet no direct evidence for imprinting of the $G_s\alpha$ transcript in humans, there is evidence that the human XLαs and NESP55 transcripts are oppositely imprinted in humans, with XLαs expressed only from the paternal allele and NESP55 expressed only from the maternal allele[26)27)]. Consistent with this, the region containing the XLαs-specific upstream exon is methylated only on the maternal allele while the NESP55-specific upstream exon is methylated only on the paternal allele[26)27)]. We have found a similar

pattern of imprinting of XLαs and NESP55 in mice (S. Yu, L. Weinstein, unpublished observations). It seems unlikely that XLαs plays a major role in the pathogenesis of AHO since its expression would only be disrupted in PPHP patients, and these patients do not have any specific phenotypic abnormalities which are not also present in PHP Ia patients (in whom XLαs expression would be unaffected). It is also unclear what role, if any, NESP55 has in the pathogenesis of AHO. There is no evidence that patients with exon 1 mutations (which should affect only $G_s\alpha$ expression) and exon 2-13 mutations (which should affect expression of $G_s\alpha$, XLαs, and possibly NESP55) have distinct phenotypes.

Unlike the human AHO model, in the GsKO mouse model paternal transmission of the null mutant leads to a distinct abnormal phenotype and maternal transmission leads to distinct abnormalities which are unlikely to be explained by decreased $G_s\alpha$ expression. It is possible that these phenotypes result from disruption of alternative *Gnas* transcripts which are only transcribed from the paternal and maternal allele, respectively, since all of these transcripts include exon 2. The $G_s\alpha$ gene is the first example of a gene with multiple oppositely imprinted promoters and alternative transcripts.

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